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An Ultrastructural Study of the Effects of a Polychlorinated Biphenyl (Aroclor 1254) on the White Pelican Hepatocytes

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AN ULTRASTRUCTURAL STUDY OF THE EFFECTS OF A POLYCHLORINATED BIPHENYL (AROCLOR 1254) ON THE WHITE PELICAN HEPATOCYTES

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser Date

Head, Biology Department Date

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IJS

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INTRODUCTION

Since the 1960's people have become concerned about pesticide residues and their effects on the environment including the human population.

Polychlorinated biphenyls (PCB's), one general class of residues, have been commercially manufactured in the United States since 1929.[14] They have been used in many products such as plasticizers, hydraulic fluids, lubricants, electrical capacitors, electrical transformers, vacuum pumps, heat transfer systems, wax extenders, dedusting agents, surface coatings, adhesives, printing inks, pesticide extenders and carbonless reproducing paper.[10]

Food and Drug Administration guidelines require that the maximum amount of PCB's should not exceed 0.20 parts per million (ppm) in milk (equivalent to 5.0 ppm in milk fat),[4] however, PCB levels have been found as high as 27.8 ppm.[9] Foods have been contaminated from the containers in which they are shipped or stored.[9]

Of 637 samples of human tissues studied in 18 states and the District of Columbia, 198 (31.1%) contained measurable amounts of PCB's and 125 (19.6%) contained trace amounts.[15] Samples of adipose tissue of occupationally exposed humans in Michigan have been found to contain as much as 180 ppm (fat basis).[13]

The long range effects of PCB's on humans and other organisms is not known. The objective of this study was to determine the effects of known concentrations of PCB's on white pelican hepatocytes and

to obtain quantitative information regarding the severity of these effects by electron microscopic analysis.

LITERATURE REVIEW

Many of the chlorinated hydrocarbons have produced tissue hypertrophy, certain ultrastructural changes such as proliferation of the endoplasmic reticulum (ER) and modification of certain hepatic enzymes.[1] Continued dietary exposure of rats to 100 ppm DDT resulted in characteristic liver lesions, including liver enlargement, peripheral migration of basophilic cytoplasm, hyalinization of central cytoplasm, and formation of inclusion bodies.[12] Electron microscopic examination revealed the hyalinization to be caused by proliferation of the smooth endoplasmic reticulum (SER). Dietary dieldrin levels as low as 2.5 ppm produced occasional rat liver cell changes similar to changes caused by lindane, toxaphene, and DDT.[11] These changes included centrolobular hypertrophy, peripheral migration of basophilic cytoplasmic granulations, and development of distinctive cytoplasmic inclusion bodies.

Similar results including hepatocyte mitochondrial abnormalities were observed in rats administered 2.0 and 5.0 mg/kg dieldrin for up to ten days.[8] SER proliferation and increased numbers of cytoplasmic vacuoles in liver parenchymal cells were observed in dieldrin-treated rats, mice, dogs, and monkeys;[17] these dieldrin-induced alterations were found to be reversible upon cessation of the treatment, the regression times varying with different species. Atypical mitochondria, cytoplasmic inclusions, and increased amounts of SER were also observed in rats fed DDT, dieldrin, or a combination

of the two.[6] Morphological changes of the mitochondria consisted of an increase in the number of cristae, a doubling of the outer membrane or loss of a portion of the outer membrane, and confluence of two mitochondria. Variable degrees of fatty degeneration of the hepatocytes were observed in dieldrin-treated guinea pigs.[16]

Hepatic tissue of rats fed 0.1% DDT, 0.1% PCB's, or 1.0% polychlorinated triphenyls contained numerous fat vacuoles, proliferated endoplasmic reticulum, and large concentric membrane (CMA) structures.[1] Administration of two PCB's (Aroclor 1254 and 1260) to rats produced changes visible at the light microscrope level consisting of liver cell hypertrophy, cytoplasmic inclusions, brown pigment in Kupffer cells, lipid accumulation, and adenofibrosis.[7] Ultrastructural changes observed in the above experiment included increased amounts of SER, atypical mitochondria, and occasional circumscription of lipid vacuoles by concentric membranes. Rats administered Aroclor 1242 exhibited large cytoplasmic vacuoles, fatty deposits, necrotic foci, and a marked increase of cytoplasmic volume in hepatocytes.[3]

MATERIALS AND METHODS

White nesting pelicans (Pelecanus erythrorhynchos) were collected at the rookery of LaCreek National Refuge on July 7, 1972, and transferred to cages at the South Dakota State University campus. Treatment started July 17 and ended September 26, 1972. Nine experimental birds received 100.0 mg of PCB (Aroclor* 1254), per day in their diet, while nine other birds served as controls. After ten weeks, PCB treatment ceased and the birds were stressed fourteen days by receiving one-half their normal food intake. On October 10, 1972, 50 ml of blood was removed from each bird by cardiac puncture. The birds were then killed by injection of 50 ml air into the heart.

Liver tissue was removed immediately with disposable biopsy needles** and placed in cold (4.0°C) 5.0% glutaraldehyde in 0.05 M KPO_4 buffer with 0.2% sodium thioglycolate at pH 7.4 for one to two hours. Samples were rinsed twice for 30 minutes in cold buffer with sodium thioglycolate followed by two 30-minute cold rinses in buffer without sodium thioglycolate. The tissue was post-fixed in cold 1% osmium tetroxide in 0.05 M KPO_4 for one hour. The pH of the buffered osmium solution was 7.4. The dehydration process began with 25% cold acetone for five minutes followed by a second cold 25% acetone rinse for 10 minutes, then

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*Monsanto Chemical Co., St. Louis, Missouri

**Tru-Cut (R) Travenol Laboratories Inc., Morton Grove, Ill.

one rinse with each of the following for 30 minutes: 50% acetone (cold), 75% acetone (cold), 100% acetone (room temperature). Two 30-minute rinses in 100% acetone and two 60-minute rinses completed the dehydration process.

The samples were then placed into a 1:1 mixture of 100% acetone and Bojax* for 3-4 hours in one-dram capped vials.** The lid was removed and the vial placed in a fume hood overnight. The following day liver tissues were placed into plastic Beem capsules*** and the capsules were filled with Bojax. The capsules were then placed in a 50°C vacuum oven at 15mm Hg for two hours. The Bojax was allowed to polymerize for 48 hours in a 60°C oven. Thin sections were cut with an ultramicrotone**** and a diamond knife.# Sections from 500-700 Å were picked up with uncoated 200 mesh grids.## The grids had been cleaned prior to use with 20% nitric acid. Approximately ten sections were picked up on each grid. Then the block was trimmed back 10um and ten more sections were picked up on a second grid. The same procedure was used for the third grid, etc. This procedure was used to ensure a random sampling of hepatocytes.

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*Araldite 6005 35%, EpoN 812 11%, DDSA 53%, DMP-30 1-2%

**"Titeseal" vials, Lab. apparatus Co. Cleveland, Ohio

***Beem capsules, size 00, Ernest F. Fullam, Inc.

****LKB Huxley Ultramicrotome

#4mm Diamond Knife, Rondikn Corp.

##Cat. No. 2200 200 mesh copper grids, Ernest F. Fullam, Inc. Schenectady, New York.

Sections from each group of pelicans were stained in 2% uranyl acetate for 20 minutes to 2 hours. The sections were rinsed in three changes of distilled water and allowed to air dry on filter paper. The sections were then stained with 0.5% lead citrate* (carbonate free) in 0.1N NaOH for 1-2 minutes. The lead citrate had been layered with mineral oil to prevent contamination. Each grid was rinsed in six changes of freshly boiled double glass distilled water. The sections were viewed with an electron microscope** at approximately 2,000X magnifications. At least 15 negatives were taken of each individual sample.

Quantitative Procedures

Quantitative information regarding a number of parameters was obtained by the following assumptions and procedures:

1. Hepatocyte size was calculated by measuring length and width dimensions in those hepatocytes which displayed nuclei with accompanying nucleoli. Since nuclei are usually centrally located within hepatocytes, such sections were assumed to provide measurements taken through the approximate center of hepatocytes. Ten different hepatocytes were measured in each liver sample.
2. Nuclear size was determined in each of the ten hepatocytes by measuring length and width dimensions.

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*Cat. No. 0378, Polysciences, Tydal, Pa.

**RCAEMU-3G, Northern Grain Insect Research Laboratory, Brookings, South Dakota.

3. Nucleolar size was determined in each of the ten nuclei by measuring length and width dimensions.
4. Mitochondria in each of the ten hepatocytes were counted.
5. Cristae were counted in 16 mitochondria in each of the ten hepatocytes.
6. Vacuoles, lipid, lysosomes, microbodies, or other membrane-bounded vacuoles were counted in each of the ten hepatocytes.
7. Mitochondrial size was determined by measuring length and width dimension of 16 mitochondria in each of the ten hepatocytes.
8. Analysis of variance test was used to determine significant differences in the above parameters between experimentals and controls.

RESULTS

General Architecture (Histological and Ultrastructural)

The general histological features of the normal white pelican liver resemble mammalian liver. Cords of hepatocytes one cell thick are surrounded by sinusoids with bile canaliculi located on the interfacial spaces between them (Fig. 1,2 and 5). Tight junctional specializations (Fig. 7) are commonly found between cell membranes apposed to the bile canaliculi. Bile canaliculi possess prominent microvilli projecting into the lumen (Fig. 1). An average hepatocyte is somewhat columnar having a greater length than width (Fig. 4). The nucleus is typically elliptical (Fig. 10) and, in most hepatocytes, contains at least one prominent nucleolus (Fig. 10). Heterochromatin is marginated along the nuclear membrane. The cytoplasm has an abundance of mitochondria which are usually cut in circular to elongate profiles (Fig. 3). Glycogen granules are randomly distributed or aggregated in large masses. Cytoplasmic lipid droplets are often in close contact with glycogen (Fig. 1). A few myeloid figures can be seen in controls as well as in the treated group. Lysosomes are scattered throughout the cytoplasm with an apparently higher concentration close to bile canaliculi.

Kupffer cells line the liver sinusoids (Fig. 2). Nuclei of the Kupffer cells are elongated with heterochromatin marginated along the nuclear membrane. Kupffer cells have long, thin processes which line the sinusoids. Lipid droplets are closely associated with Kupffer cells and often depress their nuclei (Fig. 2). White

blood cells, especially lymphocytes, are numerous in the sinusoids where they often outnumber erythrocytes (fig. 1,2,3, and 5). Occasionally, mitochondria can be seen within red blood cells.[2] Collagen fibers are quite evident and usually surround the sinusoids (Fig. 2). The space of Disse is evident between the basal margin of the hepatocyte and thin cytoplasmic processes of Kupffer cells. Plasma cells are especially numerous along the outer edge of cubodial cells of the bile ducts (Fig. 8).

Quantitative Studies

The data for Tables 1,2, and 3 were derived from 90 control and 80 experimentally treated pelican hepatocytes. The average cross-sectional area for hepatocytes of the treated group was 1069 um^2 compared to 876 um^2 in the control group (Table 3).

A total of 173 nuclei were measured; the average nuclear size in the treated group was 169 um^2. Average nuclear size in the control group was 161 um^2 (Table 3).

A total of 241 nucleoli were measured. The average size in the treated group was 6.5 um^2 compared to 7.1 um^2 in the control group (Table 3).

The total number of mitochondria counted in the 170 hepatocytes was 8,331 with an average of 54 per hepatocyte in the treated group and 43 per hepatocyte in the control group (Tabel 3): a difference which was significant at $p < 0.01$. Average size of a mitochondrion in the treated group was 5.6 um^2 while the control group was 5.9 um^2 (Table 3). Average number of cristae per

mitochondrion was higher in the control group, i.e., 10.3 compared to 8.2 (Table 3) which was significant at $p < 0.05$.

A total of 2,163 vacuoles, lipid, lysosomes, microbodies, or other membrane-bounded vacuoles were counted. The PCB-treated hepatocytes averaged about 14.1 vacuoles per hepatocyte cross-section. The control hepatocyte cross-section averaged about 11.5 vacuoles (Table 3).

Two pelicans of the treated group had fewer visible cell membranes than was normally encountered. Only four hepatocytes in bird number 11, and six hepatocytes in bird number 14 were useable for statistical comparison (Fig. 9, 10, and 11).

Fig. 1--Electron micrograph of liver from control pelican No. 2. Note junction of bile canaliculi (BC) in right center and numberous mitochondria (M); lipid droplet (LD); sinusoid (S); mass of glycogen (Arrow). X 9,800.

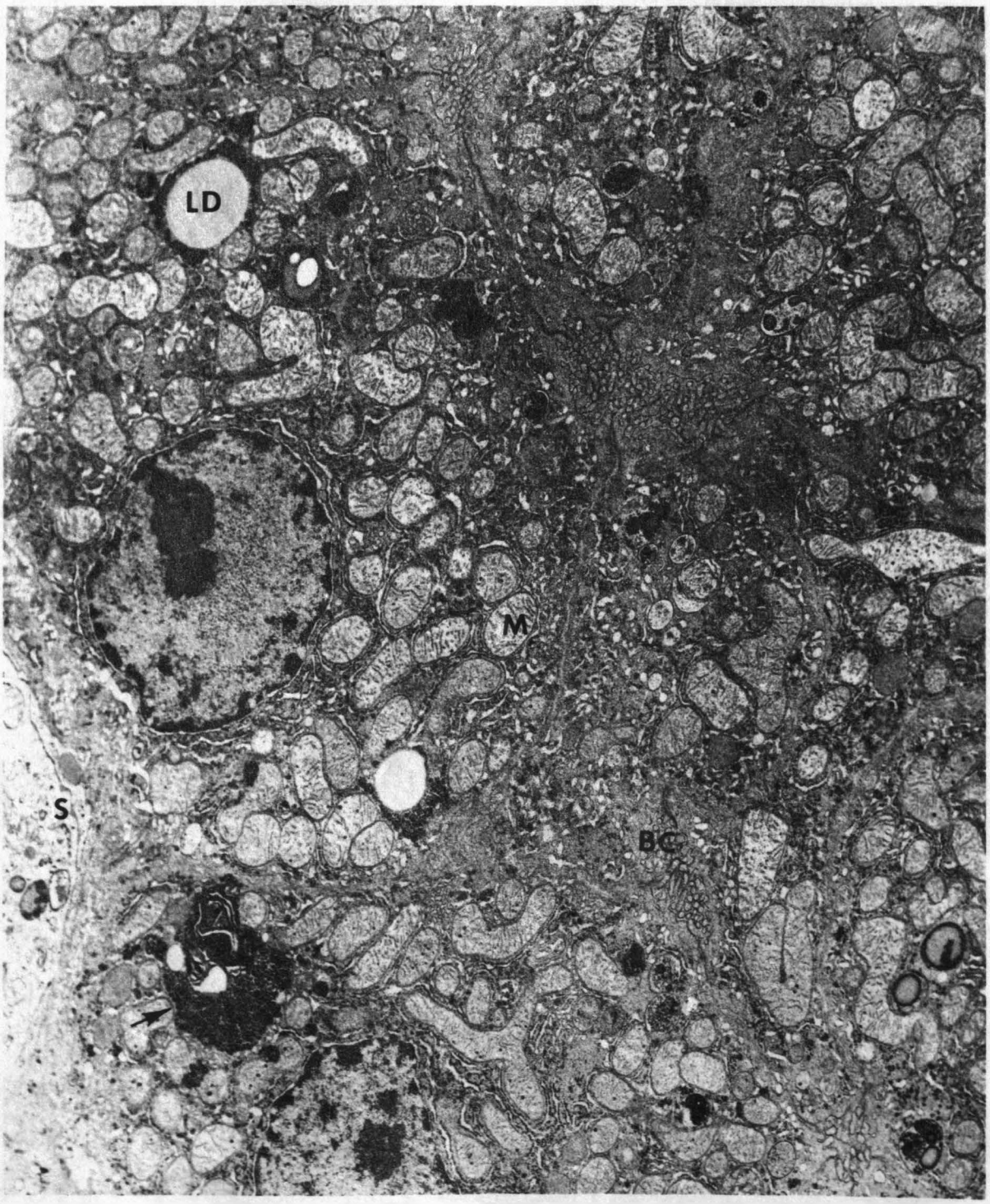
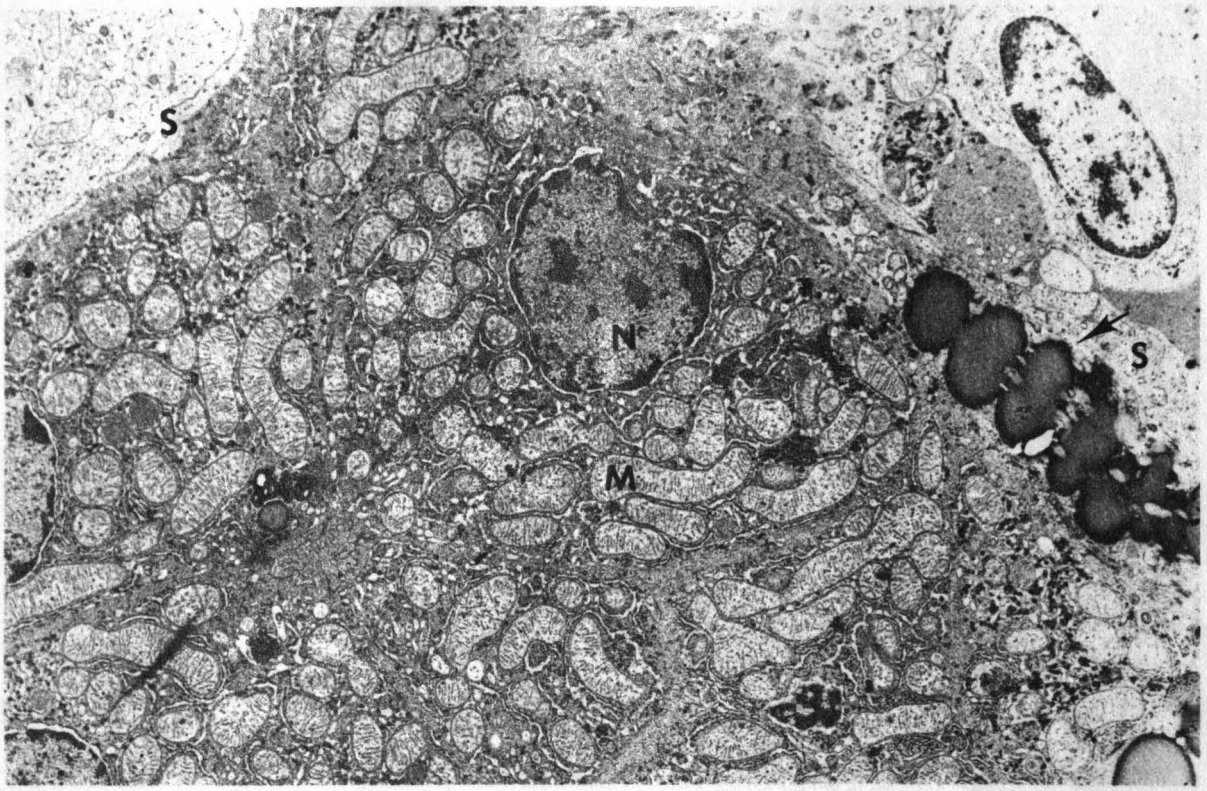


Fig. 2--Electron micrograph of liver from control pelican No. 3. Collagen (Co); spindle shaped Kupffer cell nucleus (KC); lipid droplet (LD); abnormally large mitochondrion (M) in lower left; portion of white blood cell in sinusoid (S); white blood cell (WBC); bile canaliculus (Arrow). X 10,150.


LD
KC
Co
S
M
WBC

Fig. 3--Electron micrograph of liver from control pelican No. 28. Mitochondrion (M); nucleus (N); portion of white blood cell in sinusoid (S); lipid droplets (Arrow). X 7,550.

Fig. 4--Electron micrograph of liver from PCB-treated pelican No. 9. Mitochondrion (M); nucleus (N); portion of white blood cell in sinusoid (S). X 7,500


S
N
M
S

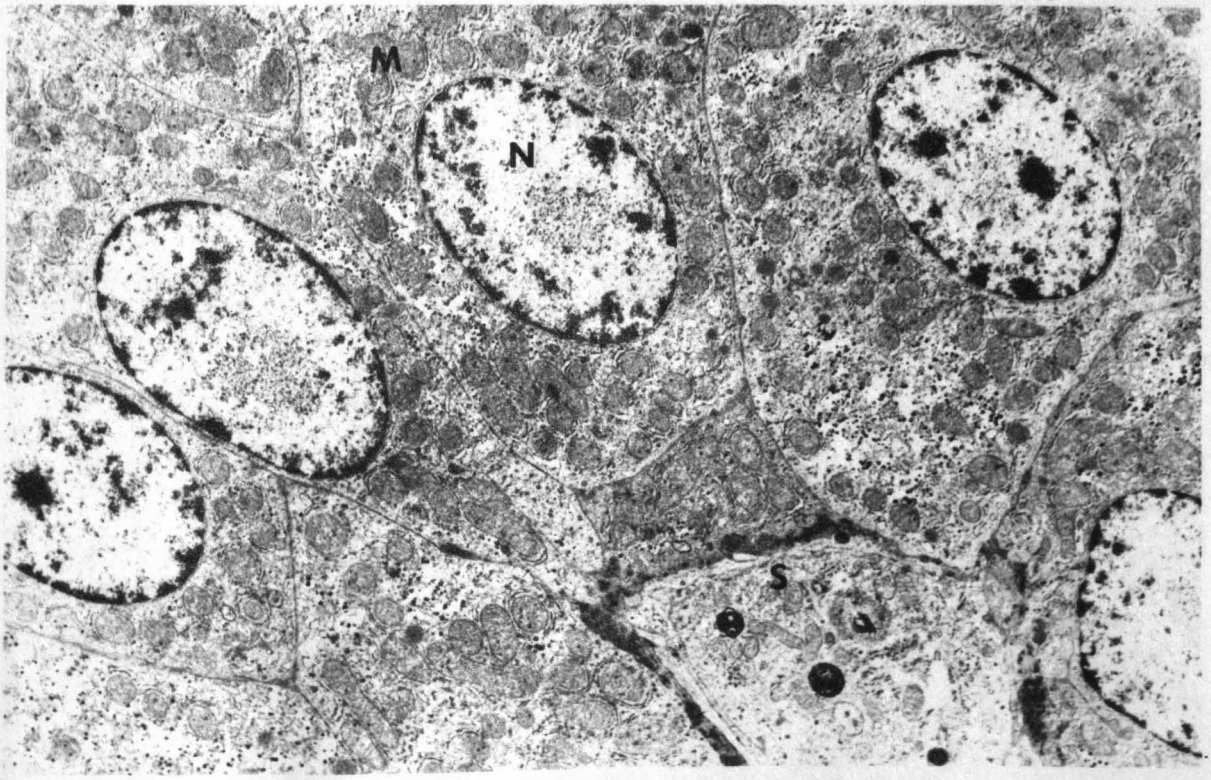

M
N
S

Fig. 5--Electron micrograph of liver from control pelican No. 8. Mitochondrion (M); portion of white blood cell in sinusoid (S); bile canaliculus (Arrow). X 7,450.

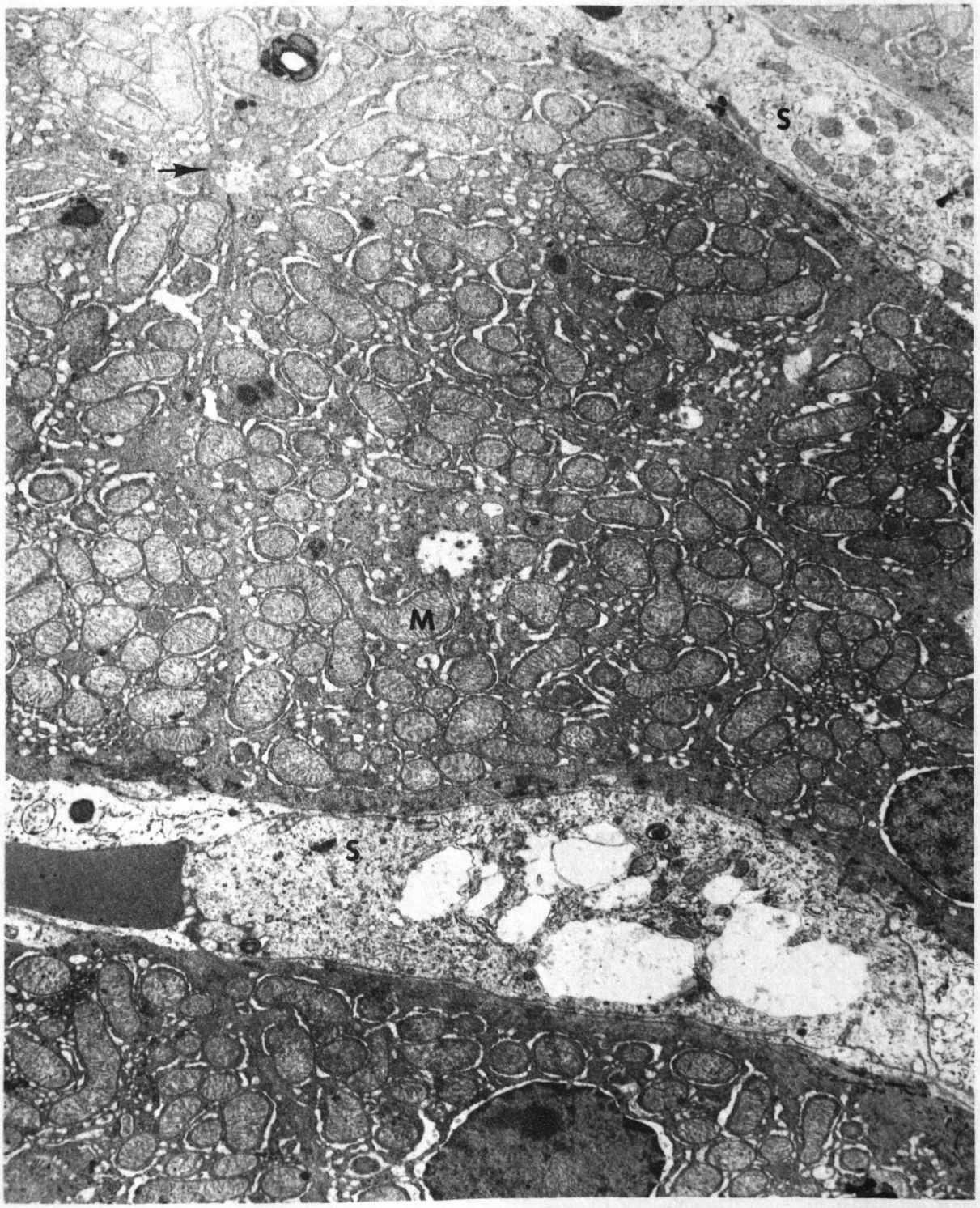
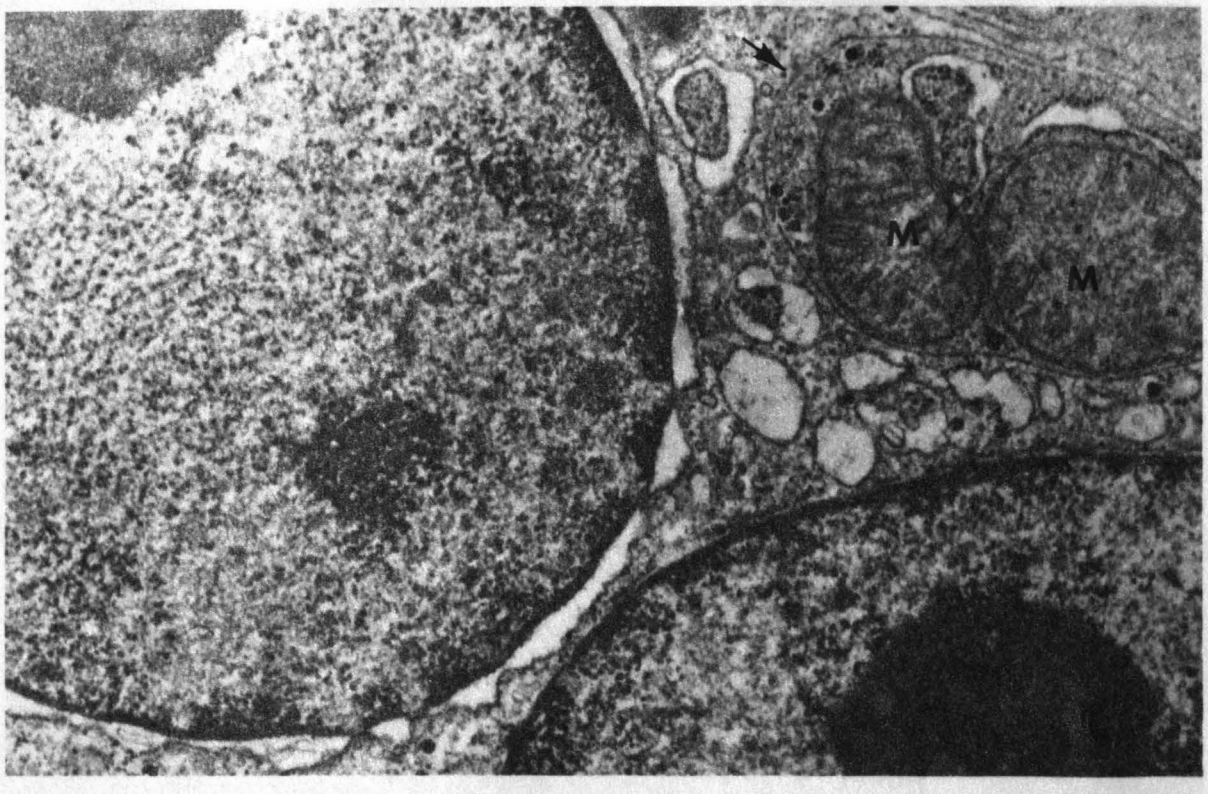

S
M
S

Fig. 6--Electron micrograph of liver from PCB-treated pelican No. 10. Arrow indicates membrane surrounding two mitochondria (M). X 27,200.

Fig. 7--Electron micrograph of liver from PCB--treated pelican No. 14. Cross-section of bile duct in right center with arrow indicating cross-section of microvillus with unidentified internal structures. Tight junctions (TJ) can be seen apposed to the bile duct. X 54,000.


M
M

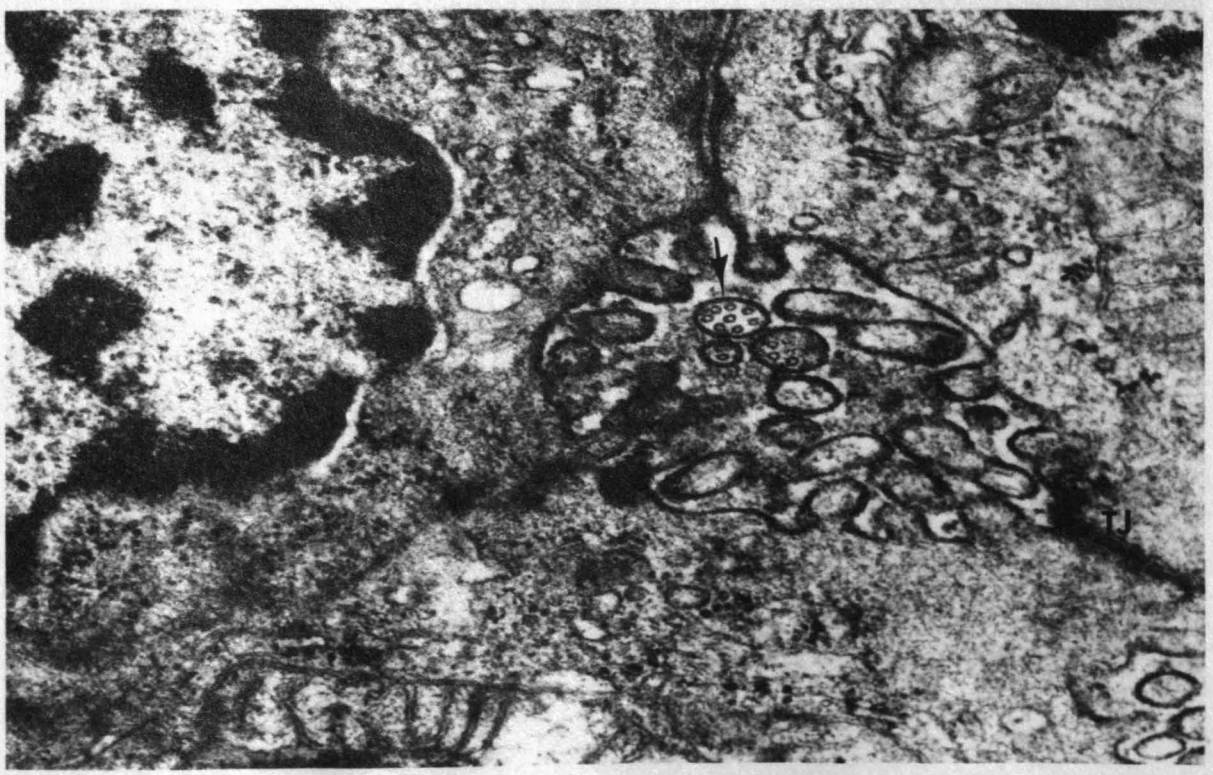

TJ

Fig. 8--Electron micrograph of liver from control pelican No. 8. Cross-section of bile duct made up of cubodial epithelial cells (C) which are surrounded by the basal lamina (BL); lumen of bile duct (Lu); plasma cell (P). X 10,000.

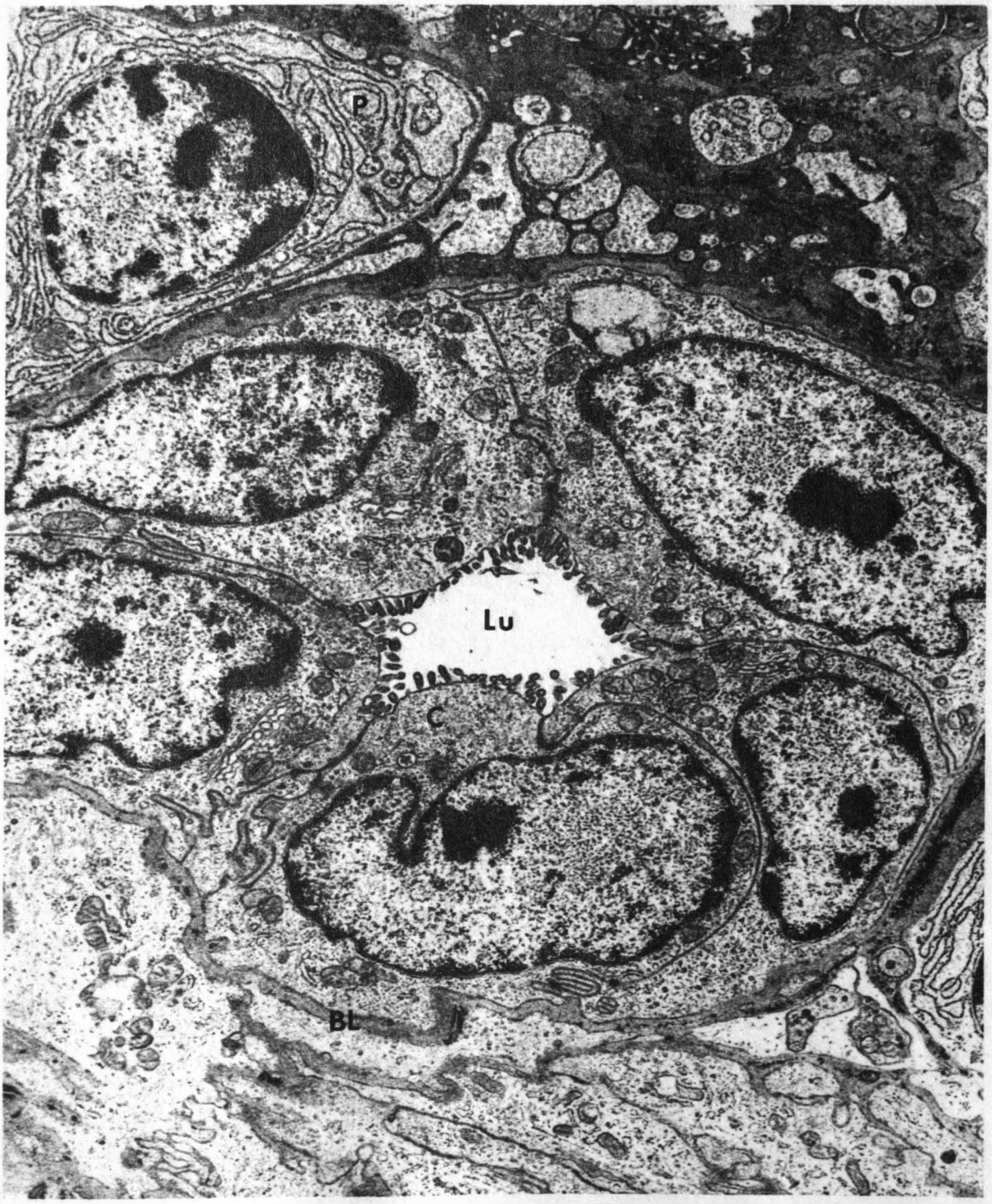
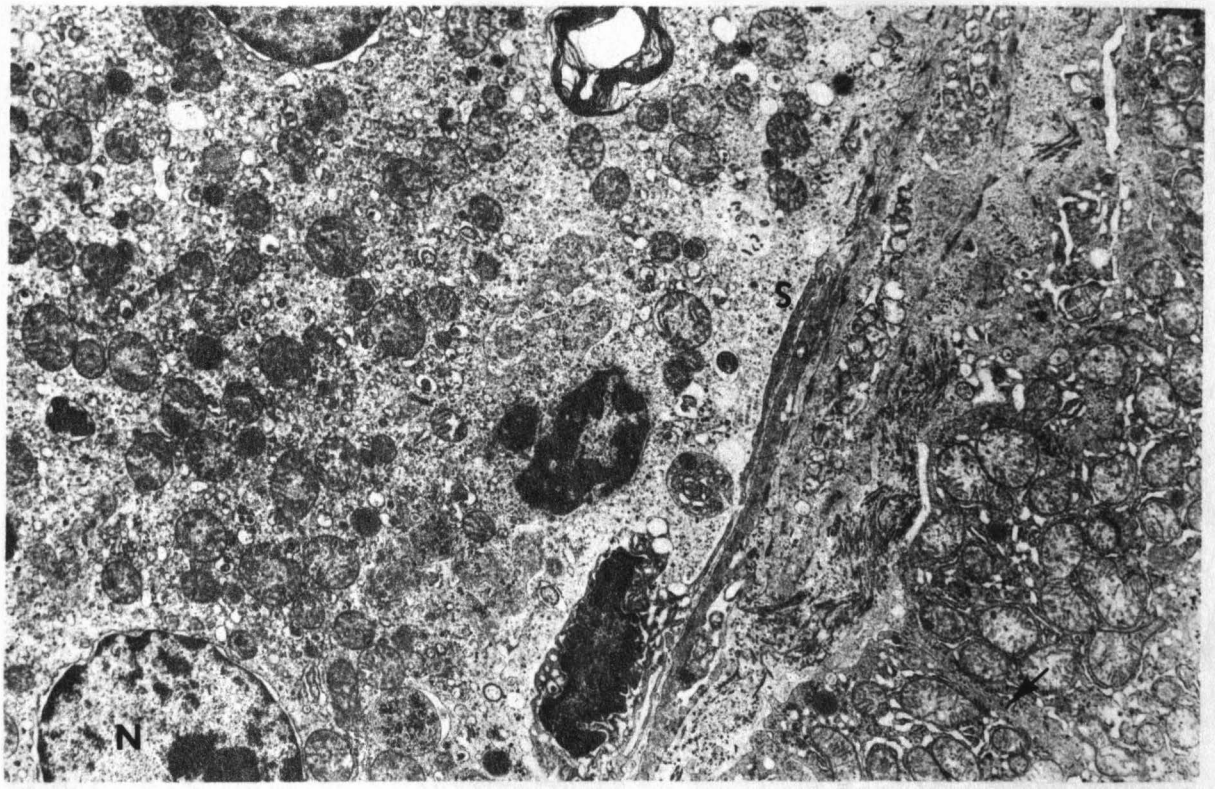

P
Lu
C
BL

Fig. 9--Electron micrograph of liver from control pelican No. 28. Note right side of sinusoid (S) appears normal while the left side of the sinusoid appears degenerate. Nucleus (N); arrow indicates cell membrane of hepatocyte. X 7,550.

Fig. 10--Electron micrograph of liver from PCB-treated pelican No. 14. Note lack of visible cell membranes of hepatocytes. Nucleus (N); nucleolus (Nu). X 11,350.


S
N

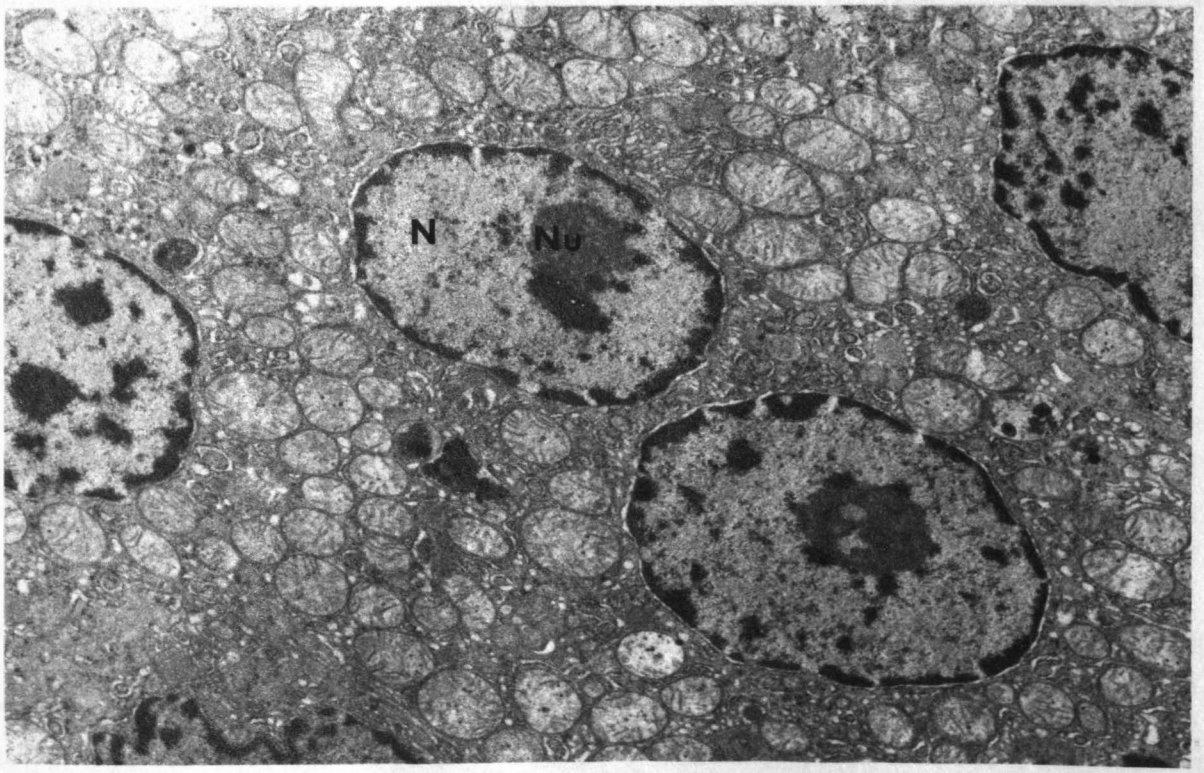

N
Nu

Fig. 11--Electron micrograph of liver from control pelican No. 2. Note degenerate appearance of hepatocytes. Nucleated erythrocyte (Arrow); lipid (L); mitochondrion (M); nucleus (N). X 9,775

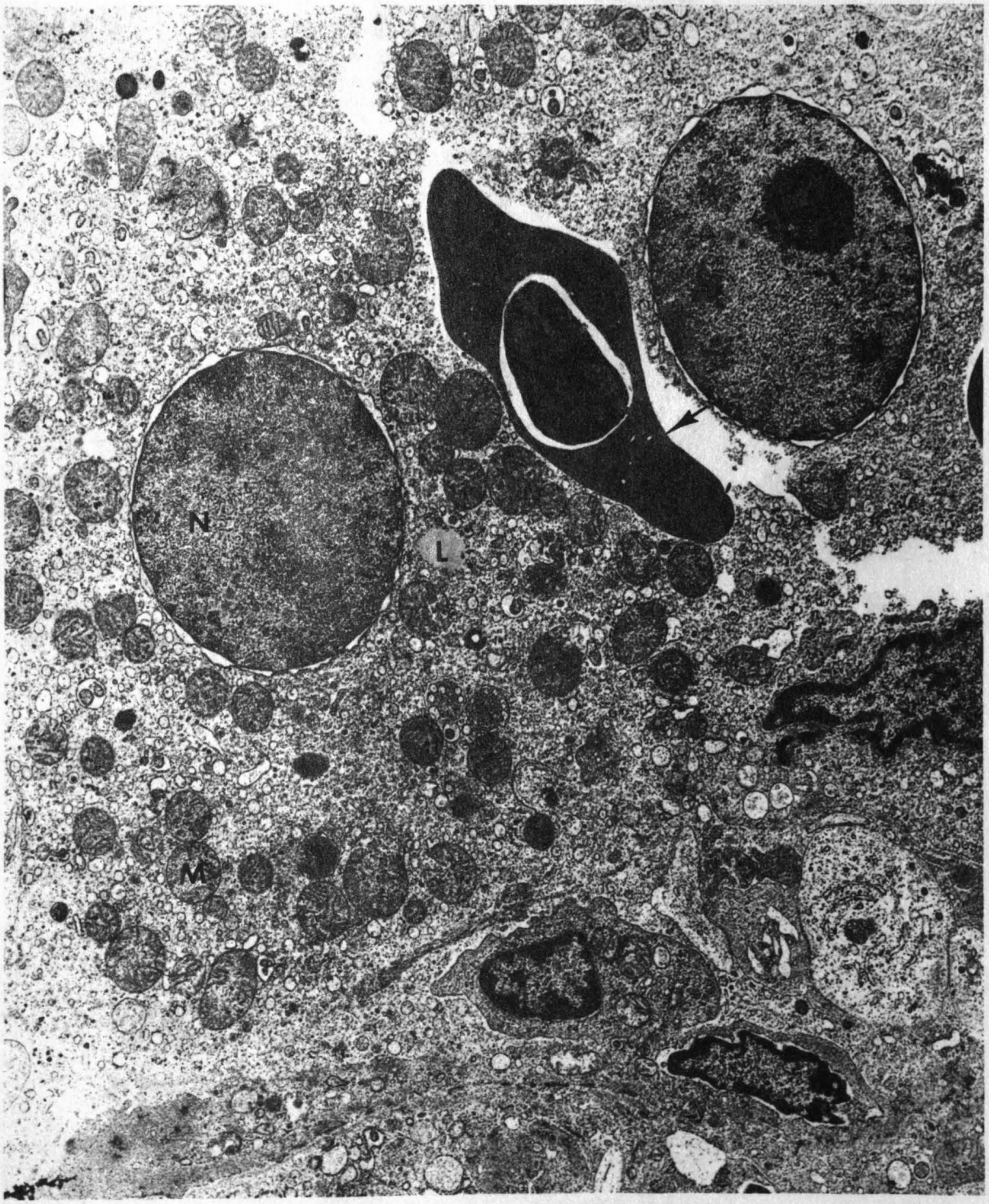

N
L
M

Table 1--Analysis of variance (F test) from hepatocytes of white pelicans treated with PCB's and controls.

Variable	Values of F		Source	Degrees Freedom	Mean Squares
Hepatocytes	1.96		total	168	137559.919
			treat	1	1271833.139
			birds	16	631331.727
			error	151	77727.985
Nuclei	0.02		total	168	4023.779
			treat	1	313.492
			birds	16	19627.286
			error	151	2394.998
Nucleoli	0.62		total	168	40.087
			treat	1	32.379
			birds	16	51.746
			error	151	38.902
Number of mitochondria	12.07	**	total	168	25145.822
			treat	1	586006.314
			birds	16	47675.223
			error	151	19044.293
Size of mitochondria	0.60		total	168	1.957
			treat	1	7.297
			birds	16	11.886
			error	151	0.870
Number of cristae	8.39	*	total	168	5.119
			treat	1	170.711
			birds	16	19.823
			error	151	2.465
Vacuoles	3.09		total	168	32.740
			treat	1	270.341
			birds	16	85.569
			error	151	25.568

** Treated group significantly different from controls at $p < 0.01$.

* Treated group significantly different from controls at $p < 0.05$.

Table 2--Numbers of hepatocytes and subcellular structures examined in livers of white pelicans treated with PCB's and controls.

	Control	Treated	Total
Hepatocytes measured	90	80	170
Nuclei measured	91	82	173
Nucleoli measured	126	115	241
Mitochondria (total)	3,888	4,443	8,331
Mitochondria measured	1,449	1,229	2,678
Cristae counted	1,428	1,225	2,653
Vacuoles counted	1,040	1,123	2,163

Table 3--Average area of cross-section or number per cross-section of various parameters tested in white pelican hepatocytes: control vs PCB treated.

	Control	Treated	% Difference between groups	Standard deviations Control	Treated
Cross-sectional area of hepatocytes	876.0 μm^2*	1069.0 μm^2*	18	340.43	383.83
Cross-sectional area of nucleus	161.0 μm^2	169.0 μm^2	4	65.54	61.38
Cross-sectional area of nucleolus	7.1 μm^2	6.5 μm^2	7	6.02	6.47
Total number of mitochondria per cross-section of hepatocyte	43.0	54.0	20	15.12	14.34
Size of mitochondria	5.9 μm^2	5.6 μm^2	5	1.50	1.24
Number of cristae per mitochondrion	10.3	8,2	20	2.20	1.82
Number of vacuoles per cross-section of hepatocyte	11.5	14.1	18	5.13	6.06

*Area calculated as product of length times width.

DISCUSSION

Comparison of the ultrastructural morphology of treated and control hepatocytes indicated an 18% increase in hepatocyte size in the treated group (Table 3). Although the analysis of variance did not show statistical significance to this finding it is in agreement with the necropsy findings of a 15% increase in gross liver weight.[5] This correlation indicated a hypertrophic change rather than a hyperplastic condition. If a hyperplastic condition did exist, it was minimal. An increase in gross liver weight and a hypertrophic condition were also found in PCB treated rats.[6]

Total numbers of mitochondria per hepatocyte indicated a significant increase (20%) in the treated group (Table 3). Analysis of variance for numbers of mitochondria between the two groups was significant at the 1% level. The treated group had slightly smaller mitochondria (Fig. 4) which appeared rounded and swollen instead of long and slender as in the control hepatocytes (Fig. 3). The finding of atypical mitochondria in this study were not in agreement with earlier findings which showed fewer mitochondria,[2] confluence of two mitochondria, double outer membrane, or loss of a portion of the outer membrane.[4,5,6] In the present study only once were two mitochondria observed within a single membrane (Fig. 6).

The number of cristae per mitochondrion were reduced 20% in the treated group; analysis of variance indicated this

finding to be significant at the 5% level (Table 3). Previous researchers indicated an increase in cristae of mitochondria within hepatocytes of rats treated with dieldrin, DDT, or a combination of the two.[7] Figure 4 shows an electron-lucent appearance of cytoplasm which was seen occasionally in some of the hepatocytes of the treated group; this is in agreement with earlier researchers.[7]

The numbers of vacuoles, lipid droplets, lysosomes, and microbodies between the two groups increased 18% in the treated group; however, analysis of variance indicated no significant difference between the two groups (Table 3). Previous researchers found an increase in at least one of the following: vacuoles, lipid droplets, lysosomes, or microbodies. These increases were noted in mice, dogs, monkeys,[14] guinea pigs,[13] rats,[1,2,4,5] and Japanese quail,[2] treated with DDT, dieldrin, PCB's, Kepone, and polychlorinated triphenyls. Pelicans in both groups of this study had lipid droplets (Fig. 1,2,3) along sinusoids or within Kupffer cells. Cytoplasmic lipid and sinusoidal lipid of the hepatocytes did not appear to vary between groups. Lysosomes were somewhat more numerous in the treated group as indicated by quantitative data (Table 3). This finding is in agreement with the work on Japanese quail.[2]

There was no noticeable increase in collagen or SER between the two groups. This has been found in rats treated with DDT, dieldrin, and PCB's.[6,7] The glycogen content of the hepatocytes

varied to such an extent that it was not possible to indicate an increase or a decrease between the two groups. No statistical changes were noted in the plasma cells, SER, and rough ER (RER) between the two groups.

CONCLUSION

This study has shown a hypertrophic effect on hepatocytes of the white pelicans treated with Aroclor 1254. Atypical mitochondria with fewer cristae ($p < 0.05$) were noted in treated pelicans along with a statistically significant increase ($p < 0.01$) in total mitochondrial numbers. There was an 18% increase in lysosomes of the hepatocytes in the treated group. No noticeable changes were seen with lipid, SER, RER, collagen, plasma cells, or Kupffer cells.

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